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Identification and Characterization of Bacteria Isolated From the Gut of Metal Treated Earthworms

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1. INTRODUCTION

Soil pollution is an important environmental problemassoil is the major sink for a variety of toxicants being released into the environment.Heavy metals released into the environment tend to persistfor a long time, as they do not undergo microbial or chemical degradation.They accumulate throughout the food chain, thus posing a serious threat to the environment, animals and humans.Thus, heavy metal resistance is a general demand of every living cell.

The common heavy metal contaminants found in the soil areCadmium (Cd), copper (Cu) and zinc (Zn). Cadmium is one of the largest three heavy metal poisons and in the body,it is known to affect several enzymes. Copper, being the third most used metal in the world,isessential for the body, but in high doses it can cause anemia, liver and kidney damage, stomach and intestinal irritation.Zinc is relatively harmlesswhen compared to several other heavy metal ions. It is important in forming complexes such as zinc fingers in DNA and as a component in cellular enzymes (Nies, 1999).Zinc plays a significant role in cytotoxic events and is prominently involved in cell death in the brain. The free zinc ion in solution is highly toxic to plants, invertebrates, and some fish(Plum *et al*, 2010).

Assessing pollutants in different components of the ecosystem and using indicator species to estimate the levels of contaminants in different parts of the ecosystem have been developedin preventing risk to natural life and public health.The earthworms have been proved to be sensitive indicators of contaminated soils. They are eco-system engineers and have been involved in bioremediation for many years.Endogeic earthworms like *Pontoscolexcorethrurus* are a major component of soil faunal communities in ecosystems of the tropics (Lavelle *et al.*, 1992).They bio-accumulate the heavy metals, detoxify them and improve the quality of soil (Sturzenbaum*et al.*,1999) (Spurgeon and Hopkin ,1999)(Nahmani*et al.*,2007). The heavy metals are taken up by the earthworms either by immobilization in the cells of gut wall or by storing in waste nodules formed within body cavity or by excretion through calciferous glands (Andersen and Laursen ,1982).

Earthworms consume large quantities of soil and thereby disperse microorganisms. Earthworms have many complex interrelationships with microorganisms and harbor millions of microbes in their gut. They depend upon microorganisms forproduction of degradative enzymes to decompose organic matter, nitrogen fixation(Morgan and Morgan, 1999;Kumar*et al*, 2010).But whether these microbes harbored by the earthworms have any significant role in detoxification process is still not very clear and is yet to be found out.

Microorganisms are ideally used in bioremediation because they possess enzymes that allow them to use environmental contaminants as food. Due to the selective pressure from the metal in the growth environment, microorganisms have evolved various mechanisms to resist the heavy metal stress. Tolerance and removal of toxic heavy metals have been studied in bacteria (Silver and Phung, 2005) and algae (Feng and Aldrich, 2004). Bioaccumulation of heavy metals is the retention and concentration of heavy metals by microbes. The heavy metal ions are transported across the microbial cell membrane from the outside to the inside of the cell where they are concentrated. In bio sorption, heavy metal ions, especially the positively charged metal ions, are sequestered through the adsorption of metals to the negative ionic groups of cell surfaces (Terry and Stone, 2002; Akhtar*et al*., 2008; Liu *et al*., 2004; Malik, 2004).

This study investigates the interaction between the endogeic earthworm *Pontoscolexcorethrurus* and their gut micro flora in uptake of heavy metals from the polluted soil samples. The endogeic earthworms have been selected for the study because these earthworms live and feed in the mineral soil layers. Hence these earthworms are in constant contact with the polluted soil and are directly influenced by the presence of metal pollutants in the soil. This study will be helpful to characterize the microorganisms harbored in earthworm's gut that take part in uptake/detoxification of heavy metal pollutants. This knowledge can further be applied to field studies to increase the efficiency of bioremediation of soil.

2. MATERIALS AND METHODS

2.1 Collection and exposure of *Pontoscolexcorethrurus* earthworms to heavy metals Cadmium, Copper and Zinc

Earthworms (*Pontoscolexcorethrurus*) were collected from the garden soil and maintained under normal conditions for a period of two weeks. To maintain uniform initial conditions in the gut, the gut of the earthworms was cleared by allowing them to feed on wet filter paper inside a box for a period of 48 hours.

The concentrations of cadmium (Žaltauskaitė and Sodienė, 2010), copper (Spurgeon*et al*, 2004) and zinc (Lev *et al*, 2010) in the soil, exposed to the earthworms were found out from reviewing the literature.Six earthworms of uniform size were weighed (day 0) and introduced to increasing concentrations of cadmium (0,40,80,160, 320 and 640mg/kg dry wt. of soil), copper (0, 100, 200, 400, 800, 1600 and 3200mg/kg dry wt. of soil) and zinc(0, 400, 800, 1600, 3200 and 6400 and 13200mg/kg dry wt. of soil) in triplicates.The soil without any addition of heavy metal was used as control.The earthworms were maintained for 15 days with adequate aeration, moisture content and were regularly monitored for percentage of weight gain/loss and mortality on days 3, 7, 10, 12 and 15.The earthworms that survived the metal treatment were used for isolation of gut micro flora.

2.2 Isolation of microorganisms from the gut of earthworms subjected to heavy metal treatment

The earthworms that survived the heavy metal treatment for 15 days were used for isolation of the gut micro flora. They were surface sterilized and the region below the gizzard (Intestine) was divided into three parts- anterior, middle and posterior parts of the intestine. The gut homogenate of control earthworms and metal treated earthworms was prepared. A diluted sample of homogenate was spread plated on the nutrient agar plates to obtain pure cultures.

2.3 Biochemical characterization and identificationof bacteria isolated from the gut of earthworms subjected to heavy metal treatment

The bacterial colonies obtained, were characterized using different biochemical assays like Gram Staining, IMViC Test, Catalase test, Oxidase test, Urease test, Nitrate reduction test,Mannitol agar test, Hydrogen sulphide production Test, Endospore forming test and Sugar fermentation assays for the identification of the species (Cappuccino and Sherman.,2004).The microbes isolated from the gut of earthworms subjected to metal treatment were compared with the gut micro flora of control earthworms to identify the bacteria that were specifically harbored in the gut of earthworms subjected to heavy metal treatment.

The various microorganisms that were selectively accumulated in the gut of metals (Cd, Cu and Zn) treated earthworms were identified using 16Sr RNA sequencing technique. This was done by outsourcing to Serene Biosciences, Bangalore.

2.4 Analysis of heavy metal concentration in soil samples using Atomic Absorption Spectroscopy (AAS)

The ability of *Pontoscolexcorethrurus*earthworms to bio-accumulate the heavy metals in the soil was assessed using Atomic Absorption Spectroscopic studies in the soil samples, used before and after the growth ofearthworms.

A known concentration (ppm) of cadmium chloride, copper chloride and zinc chloride were added to respective boxes where *Pontoscolexcorethrurus*earthworms were grown for a period of 15 days. About 2 grams of soil sample was collected from each box used for the growth of earthworms under metal stress ($CdCl_2$, $CuCl_2$ and $ZnCl_2$), after the introduction of earthworms and were processed in triplicates for each metal concentration according to standard procedures of AAS sample preparation. The samples were analyzed for the quantity of heavy metals using AAS, which was outsourced to Chemical Laboratory, Department of Mines and Geology, Government of Karnataka.

2.5 Characterization of isolated microorganisms based on their tolerance to heavy metals,Antibiotic Sensitivity andresistance to Herbicides using agar diffusion assay

Agar Well Diffusion assay was performed to study multi metal tolerance of the isolated bacterial cultures. Petri plates containing 20ml Luria Bertani medium were seeded with 100 µl of overnight grown culture of bacterial isolates.Stock solutions of copper chloride, zinc chloride, cadmium chloride were prepared and introduced into wells so as to obtain required concentration of 4µg/well, 16µg/well, 32µg/well, 64µg/well, 128µg/well and 256µg/well of the metals. The plates were incubated to observe the microbial growth. The assay was conducted in four trials and multi metal tolerance was assayed by measuring the diameter of the inhibition zone formed around the well in centimeters (NCCLS, 1993).

The sensitivity of the isolated bacterial species to different antibiotics was evaluated by agar well diffusion method.Four antibiotics tablets namely Ampicillin, Tetracycline, Streptomycin and Kanamycin were placed at equidistant places from each other in the LB agar plates.The test was carried out in triplicates. The plates were incubated and the zone of inhibition for each was recorded.

As the study was carried out on earthworms harboring soil microorganisms, the ability of soil bacteria to resist herbicides was studied using agar well diffusion technique.The normal concentration generally sprayed and applied in fields for these herbicides was 10ml/l for glyphosate (liquid), 1.25g/l for 2, 4, Dichlorophenoxyacetic acid (powder form) and 2.67g/l of atrazine (powder form). Ten times higher and ten times lower concentrations of these herbicides were used to check the resistance of these four bacterial strains.Different stock concentrations of the herbicides were prepared and introduced into the wells to obtain required concentration of the herbicides. The plates were then incubated and the zone of inhibition was recorded in centimeters.

2.6 Characterization of microorganisms for differential expression of metal resistant proteins using SDS PAGE

All the isolated organisms were inoculated in 3 ml of LB broth with 128µg of different metals used in the study and incubated overnight.Crude protein extracts were prepared from the bacterial samples and was loaded into Polyacrylamide gel with 12.5% of resolving gel and 5% of stacking gel and electrophoresed. The gel was stained in coomasie blue staining solution (Laemmli, 1970) and analyzed for the presence of differentially expressed proteins under the influence of metals compared to the control conditions.

2.7 Characterization of microorganisms based on the presence of plasmids conferring metal resistance

The bacterial pellet obtained from 3ml of overnight grown culture of the bacterial strains was subjected to alkaline lysis method (Birnboim and Doly 1979). Each isolated sample was loaded on 1% agarose gel and electrophoresed to look for the isolated plasmid DNA.

3. RESULTS AND DISCUSSION

3.1 Exposure of *Pontoscolexcorethrurus*earthworms to heavy metals Cadmium, Copper and Zinc

Cadmium had a deleterious effect on growth of the earthworms. All theearthworms that were introduced in the soil spiked with Cadmium salt, irrespective of its concentration, showed decrease in their weights by day 3. The earthworms exposed to concentrationof Cadmium more than 80mg/kg were found dead within 8 days (Table1).

There was a gradual weight loss of earthworms, observed under the effect of Copper. The earthworms exposed to a concentration of more than 1600mg of copper /kg of dry weight of soil, died within three days and there were no earthworms surviving at or above 800mg/kg at the end of the fifteen-day period (Table 2).

Pontoscolexcorethrurus earthworms were able to tolerate zinc chloride up to a concentration of 1600 mg/kg of dry weight of soil. All the earthworms introduced in the soil spiked with zinc salt, irrespective of its concentration, showed variation in their weights by day3. The earthworms exposed to concentration of zinc more than 1600mg/kg were found dead within 6 days (Table 3)

Table 1 Effect of Cadmium on the growth of earthworms, *Pontoscolexcorethrurus*.

	Average weight & percentage weight gain/ loss on the days									
	0^{th}day		3^{rd}day		8^{th}day		10^{th}day		14^{th}day	
Concentration of $CdCl_2$ (mg/kg of soil)	g[a]	%[b]	g[a]	%[b]	g[a]	%[b]	g[a]	%[b]	g[a]	%[b]
0	0.19(18)	100	0.19(17)	0	0.23(17)	+21	0.22(16)	+15.8	0.2(14)	+5.3

40	0.16(18)	100	0.14(18)	-12.5	0.14(15)	-12.5	0.15(12)	-6.2	0.17(11)	+6.3
80	0.14(18)	100	0.12(18)	-14.3	0.11(12)	-21.4	0.12(11)	-14.3	0.13(05)	-7.1
160	0.13(18)	100	0.1(15)	-23.1	0	0	0	0	0	0
320	0.13(18)	100	0.09(12)	-30.8	0	0	0	0	0	0
640	0.15(18)	100	0.08(11)	-46.7	0	0	0	0	0	0

Table 2Effect of Copper on the growth of earthworms, *Pontoscolexcorethrurus*

	Average weight & percentage weight gain/ loss on the days											
	0th day		3rd day		7th day		10th day		13th day		15th day	
Conc.of $CuCl_2$ (mg/kg of soil)	g[a]	%	g[a]	%	g[a]	%	g[a]	%	g[a]	%	g[a]	%
0	0.17 (18)	100	0.27 (17)	58.8	0.19 (15)	14.3	0.16 (15)	-5.8	0.17 (13)	0	0.17 (13)	0
100	0.18 (18)	100	0.24 (16)	33.3	0.19 (15)	5.5	0.15 (13)	-14.5	0.15 (13)	-23.8	0.13 (11)	-34.6
200	0.18 (18)	100	0.23 (18)	28.4	0.18 (17)	0	0.14 (17)	-24.8	0.15 (17)	-15.5	0.13 (15)	-28.8
400	0.18 (18)	100	0.23 (18)	27.4	0.17 (17)	-4.3	0.15 (14)	-11.2	0.14 (14)	-16.9	0.14 (11)	-19.1
800	0.19 (18)	100	0.21 (09)	23.3	0.17 (04)	-5.8	0.10 (03)	-16.8	0	0	0	0
1600	0.16 (18)	100	0.16 (10)	0	0	0	0	0	0	0	0	0
3200	0.21 (18)	100	0	0	0	0	0	0	0	0	0	0

Table 3Effect of zinc on the growth of earthworms, *Pontoscolexcorethrurus*

	Average weight & percentage weight gain/ loss on the days									
	0thday		3rdday		8thday		10thday		14thday	
Conc. of $ZnCl_2$ (mg/kg of soil)	g[a]	%[b]	g[a]	% [b]	g[a]	% [b]	g[a]	% [b]	g[a]	% [b]
0	1.12(18)	100	1.47(18)	+30.6	1.72(18)	+53.0	1.30(18)	+16.2	1.49 (18)	+32.8
100	0.97(18)	100	1.44(18)	+47.5	1.29(18)	+32.2	1.31(18)	+34.0	1.2(18)	+22.6
400	0.86(18)	100	0.97(15)	+12.1	1.03(15)	+19.3	0.95(15)	+10.0	0.93(15)	+8.1
800	0.61(18)	100	0.77(18)	+25.3	0.61(18)	0	0.61(18)	0	0.6(18)	-1.6
1600	0.59(18)	100	0.57(18)	-3.3	0.63(15)	+6.77	0.63(15)	+6.77	0.59(15)	0
3200	1.08(18)	100	1.02(3)	-5.5	0	0	0	0	0	0
6400	0.74(18)	100	0	0	0	0	0	0	0	0
13200	0.65(18)	100	0	0	0	0	0	0	0	0

a: Average weight of the earthworms alive on the day of observation. The number in parentheses indicates the number of the earthworms alive on the day of observation.
b: Percentage change in the average weight of the earthworms compared to the 0^{th} day.

3.2 Biochemical characterization and identification of bacteria isolated from the gut of earthworms subjected to heavy metal treatment

Depending upon the Gram's natureand morphology of the micro organisms, they were subjected to a series of different biochemical tests.

*Delftia*sp.and *Staphylococcus aureus* were bacteria that were enriched or selectively accumulated under in the gut of earthworms subjected to cadmium treatment.*Staphylococcus aureus*and *Bacillus cereus*were found to be selectively accumulated in the gut of earthworms exposed to copper treatment.When the bacteria isolated from the gut of earthworms subjected to zinc treatment were compared with the gut micro flora of control earthworms, *Aeromonashydrophila*and *Bacillus cereus* were found to be selectively accumulated in the gut of earthworms exposed to zinc stress.The microorganisms that were selectively accumulated in the gut of *Pontoscolexcorethrurus* earthworms on metal treatment were identified using 16Sr RNA sequencing technique. This was done by outsourcing to Serene Biosciences, Bangalore .

3.3 Analysis of heavy metal concentration in soil samples using Atomic Absorption Spectroscopy (AAS)

AAS was carried out to find the cadmium, copper and zinc concentrations in soil samples used for the growth of earthworms and after the growth of *Pontoscolexcorethrurus* earthworms. When the results were statistically analyzed using student T test, it showed a significant difference at ($p < .05$) (95%) indicating drastic decrease in the concentration of these heavy metals after the growth of earthworms. This study proved the ability of *Pontoscolexcorethrurus*earthworms to bio-accumulate the heavy metals like cadmium, copper and zinc present in the soil.

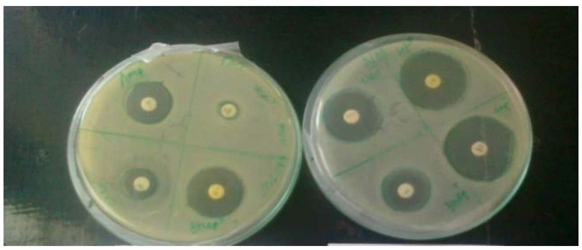
3.4 Characterization of isolated microorganisms based on their tolerance to heavy metals Antibiotic Sensitivity andresistance to Herbicides using agar diffusion assay

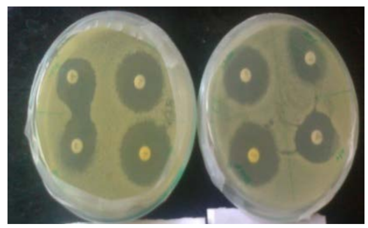
These isolated microorganisms, *Staphylococcus aureus, Delftia* sp., *Bacillus cereus* and *Aeromonas sp.* were assessedbased on their ability to tolerate different concentrations of heavy metals ($CdCl_2$, $CuCl_2$ and $ZnCl_2$), antibiotics and herbicides.

*Staphylococcus*sp., showed zone of inhibition in all concentrations of cadmium chloride indicating that the organism was sensitive towards cadmium even at lower concentration of 4µg/well.However, the organism was able to tolerate Cu even at higher concentrations up to 256µg/well. In case of zinc chloride, no inhibition was observed even up to 64µg/well indicating that the organism could tolerate or resist the metal at that concentration .*Delftia*sp.was able to tolerate Cd better when compared to *Staphylococcus* and *Bacillus*. The organism was able to tolerate Cu even at high concentrations of 256µg/well.*Delftia*sp.showed tolerance toZinc up to 128µg/well.*Bacillus*was more sensitive towards Cd as was indicated by increased zone of inhibition. Bacillus was able to only tolerate Zn and Cu even at high concentrations of 256µg/well.*Aeromonas* was able to tolerate cadmium up to a concentration of 16μg/well.The organism was able to tolerate Cu and zinc even at concentrations of 256μg/well.It was observed that all the four bacterial strains were relatively more sensitive to cadmium however were able to tolerate copper and zinc.

The antibiotic sensitivity of the bacterial strains isolated from the gut of *Pontoscolexcorethrurus*earthworms exposed to heavy metals, were evaluated by disc diffusion method (Kirby-Bauer, 1972). All the bacteria were sensitive to all the four antibiotics, (Ampicillin, Streptomycin, Chloramphenicol and Tetracycline) showing different ranges of sensitivity (Figure1).

Fig1Zone of inhibition shown by different bacteriaagainst antibiotics





Aeromonas Staphylococcus

B.cereus Delftia

All the four organisms (*Staphylococcus*,*Delftia*,*Bacillus* and*Aeromonas*) used were found to be resistant to all the three herbicides (Glyphosate, 2, 4-D and Atrazine) used in three different concentrations respectively.

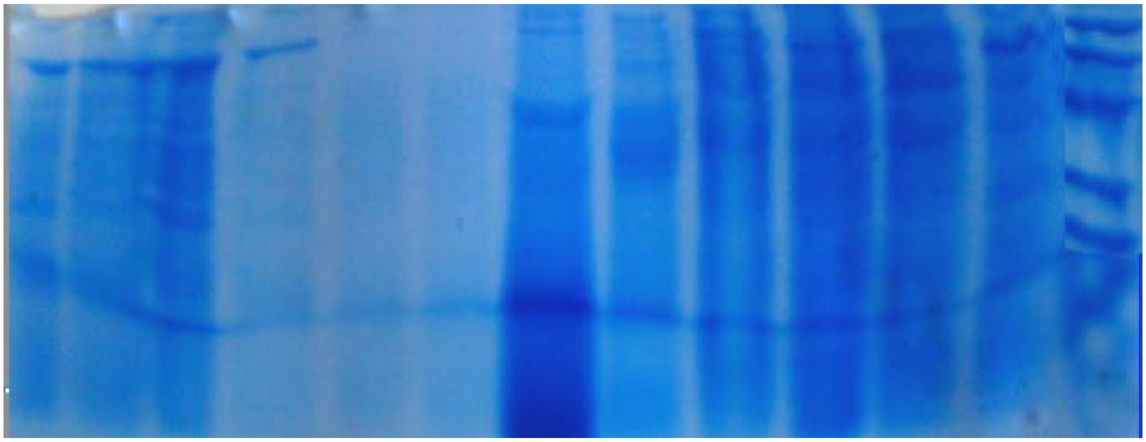
3.5Characterization of microorganisms for presence of plasmids conferring metal resistance and differential expression of metal resistant proteins

In the presence of Cadmium, all the four bacterial strains showed over expression of certain proteins compared to the control(Figure 2).In*Bacillus cereus,* when compared to control, over expression of a proteincorresponding to molecular weight of 97kDa, was observed in the lane, where protein extract from bacteria grown in the presence of cadmium was loaded.*Delftia*sp.showed over expression of three different proteins when compared to control, bearing molecular weight of approximately 43kDa, 66kDa and a high molecular weight protein >200kDa.In*Staphylococci*,a protein corresponding to a molecular weight of approximately 80kDa was over expressed, in thepresence of Cadmium, when compared to control. A high molecular weight protein >97kDa and >200kDa was also observed. In *Aeromonas*,over expression of proteins was observed in presence of Cadmium whose molecular weight was found to be 80kDa and high molecular weight protein which was more than 205kDa(figure 3).

Fig 2Analysis of crude protein extract from *Bacillus, Staphylococci and Delftia*spp. in thepresence of Cu, Cd, Zn metals

1 2 3 4 5 6 7 8 9 10 11 12 13

MW
KDa
205
97.4
66

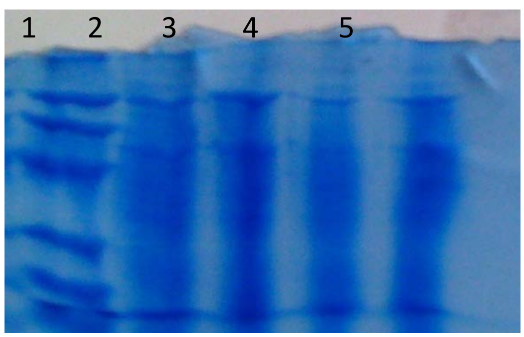


LANE1:*Bacillus*Zinc, LANE2:*Bacillus* Copper, LANE3:*Bacillus* Cadmium,LANE 4*:Bacillus* Control, LANE5*:Staphylococci*Zinc, LANE 6*:Staphylococci*Copper, LANE 7:*Staphylococci*Cadmium, LANE8*:Staphylococci*Control, LANE 9*:Delftia*Zinc, LANE 10*:Delftia*copper, LANE 11*:Delftia* cadmium, LANE 12*:Delftia* control, LANE13: Broad range Marker

<u>Fig 3Analysis of crude protein extract from *Aeromonas*sp. in thepresenceof Cu, Cd, Zn metals</u>

MwkDa

205
97.4
66
43
29



LANE 1: Broad range marker, LANE 2*: Aeromonas* Control, LANE 3:*Aeromonas*Cadmium, LANE 4:*Aeromonas* Copper, LANE5: *Aeromonas* Zinc

When the bacteria isolated from earthworms treated with heavy metals were investigated using alkaline lysis method, Plasmid DNA was not found in any of the four bacterial strains.

CONCLUSION

The present study analyzed the effect of heavy metals, cadmium, copper and zinc on the earthworms *Pontoscolexcorethrurus* and its gut microbial communities.The observed changes in the bacterial communities of the earthworms could be used as an indication for the possible contamination of the soil areas. Upon exposure to the heavy metals, the earthworms have shown enrichment of a few bacteriaby harboring them in their gut in higher amounts than in control. The presence of these selectivebacteria or enrichment of bacteria under stress conditions could imply their positive role in aidingthe earthworm’s tolerance to the metal. This also indicates an association between the earthworms and the microorganisms to bio-remediate the toxic conditions created by heavy metals. These differentially accumulated organisms may be present in the soil but there might nothave been any interaction between the earthworm and these bacteria. Upon exposure to the metal,the organisms might have been taken up and retained by the earthworms that ultimately conferredthem the resistance to the metal.

Bacteria like *Delftia*sp.and *Bacillus cereus* can be used for remediation of soils that has been polluted by different heavy metals since they are able to tolerate more concentrations of heavy metal.However, further research on the extent of tolerance and mechanism of the tolerance of heavy metals by these bacteria has to be done before their probable use in bioremediation. This will help in the understanding of the organism's capability in bioremediation and also throws light on the various methods that can be adapted to enhance this efficiency.

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